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Translational Research for Muscular Dystrophy W81XWH-11-1-0330

Gregory A. Cox, Ph.D., Principal Investigator

A. INTRODUCTION

The goal of this work is to increase the availability of critical mouse models of human muscular dystrophy (MD) for both hypothesis testing and preclinical therapy development. Our multi-disciplinary team from The Jackson Laboratory (JAX) and the Children's National Medical Center (CNMC) has expertise in MD, repository management, mouse models, and preclinical testing. At JAX, Drs. Lutz and Cox have established the MD Repository (Aim1) to leverage JAX's considerable expertise and infrastructure to maintain and distribute MD mouse and information resources to the scientific community. In Aim 2 we are developing novel DMD transgenic mice, which model patients receiving successful exon-skipping therapies. We propose to address the fundamental, but often overlooked question related to the functionality of resulting Dystrophin molecules containing in-frame deletions that are expected to arise by successful treatment of patient mutations. Our transgenic experiments will model the best-case-scenario outcome for AO-mediated therapy in which one assumes that a particular compound is capable of 100% effective exon-skipping to restore the reading frame. In Aim 3, we are generating congenic *mdx* mice to better model the symptoms of the human disease and to identify genetic modifiers that can alter disease onset and severity. In Aim 4, Dr. Nagaraju at CNMC is carrying out preclinical studies with three promising therapeutic compounds (GW501516, AICAR and Dantrolene) using key models developed at JAX. Overall, this program will greatly expand the accessibility and availability of mouse model resources for MD translational research and therapeutic development.

B. BODY

Aim 1. Develop a centralized repository for mouse models of MD. JAX.

Our goal is to identify, import, and cryopreserve 3-5 biomedically significant models per year for the MD Repository and to disseminate mouse information resources to the scientific community. The repository is providing researchers with centralized access to high-priority DMD models imported from outside investigators and transgenic and congenic DMD models developed in **Aims 2 & 3**. The following lines have been identified as relevant models to the MD Repository at JAX. We have reached out to the investigators who engineered the mice, requesting that they deposit the models to the JAX MD Repository. A description of the models and the status of the request are outlined below:

1. JAX stock 16587 B6;129-Itga7^{tm1Burk/J}.

Homozygous mice ($\alpha 7^{-/-}$) show absence of $\alpha 7$ integrin chain protein in skeletal muscle and vascular tissues compared to wildtype controls, while heterozygous mice ($\alpha 7^{+/-}$) show reduced levels. Expression of lacZ from the mutant allele is observed in $\alpha 7$ integrin-expressing cells of the embryonic vasculature, skeletal/non-skeletal musculature and central/peripheral nervous system beginning at embryonic day (E)11.5; significantly increasing out to ~E14.5. Although not detected in embryonic heart, lacZ expression is observed in adult heart tissues.

Homozygous mice exhibit myopathy, vascular smooth muscle defects, altered extracellular matrix deposition, and altered expression of other integrin chains in the cerebral vasculature. Homozygotes are embryonic lethal (with incomplete penetrance) due to vascular smooth muscle hypoplasia, cerebral vascular hemorrhaging, and reduced vasculogenesis starting ~E10.5. Of note, surviving homozygotes exhibit vascular smooth muscle hyperplasia (that may contribute to their survival). Heterozygous mice are viable and fertile, with a minor incidence of embryonic lethality. These $\alpha 7$ -mutant mice may be useful in studying cellular adhesion to the extracellular matrix,

vascular development/integrity, and the pathology of cerebrovascular and cardiovascular disease. Because laminin binding complexes ($\alpha 7\beta 1$ integrin, dystrophin and utrophin) provide continuity between laminin and the cell cytoskeleton with the extracellular matrix, these mice are used to study skeletal muscle, myopathies, and neuromuscular diseases including Duchenne muscular dystrophy (DMD).

STATUS: requested; donating investigator able to send; line successfully re-derived at JAX January, 2012 and available for distribution April, 2012.

2. JAX stock 17929 B10.Cg-Cmah^{tm1Avrk} Dmd^{mdx}/PtmJ

These Cmah-mdx mutant mice harbor two mutations; the cytidine monophospho-N-acetylneuraminic acid hydroxylase (Cmah) knockout allele (Cmah^{tm1Avrk}) and the spontaneous X-linked muscular dystrophy mutation (Dmd^{mdx}). The Cmah knockout allele abolishes Cmah expression; eliminating biosynthesis of N-glycolylneuraminic acid (Neu5Gc) from all cells and mimicking the normal human lack of functional CMAH. The Dmd^{mdx} mutation abolishes dystrophin expression and models Duchenne muscular dystrophy (DMD) with less severe myopathology.

Compared to homozygous mdx mice (Dmd^{mdx/mdx} females / Dmd^{mdx/Y} males), Cmah-deficiency in homozygous Cmah-mdx mice (Cmah^{-/-};Dmd^{mdx/mdx} females / Cmah^{-/-};Dmd^{mdx/Y} males) results in accelerated age of onset, rate of progression, and severity of the major DMD phenotypes contributing to morbidity and mortality. This is especially true for loss of ambulation, cardiac and respiratory muscle weakness, and loss of lifespan. Homozygous Cmah-mdx mice also exhibit diminished expression/function of the dystrophin-associated glycoprotein (DAG) complex and increased activation of complement. Specifically, homozygous Cmah-mdx mice have Neu5Gc accumulation into small regenerating muscles in dystrophic regions. Some homozygous Cmah-mdx mice exhibit serum antibodies to Neu5Gc and increased classical complement Neu5Gc-dependent killing of muscle cells. The donating investigator reports one can successfully breed Cmah^{-/-};Dmd^{mdx/mdx} females with Cmah^{-/-};Dmd^{mdx/Y} males if the mice are young (starting at 6-8 weeks old).

STATUS: requested; donating investigator has agreed to send; Mice have shipped to JAX in May 2012 for re-derivation.

3. JAX Stock 16622 STOCK Utrn^{tm1Jrs} Dmd^{mdx}/J

Mice homozygous for the utrophin (Utrn^{tm1Jrs}) and Dmd^{mdx} alleles have a lifespan of 4 to 20 weeks, with only 50% surviving beyond 8 weeks of age. As early as 3 weeks of age homozygotes display growth delays, are smaller than wildtype controls and exhibit skeletal muscle dystrophy with abnormal waddling gait at 4 weeks of age. At 10 weeks of age, double mutants exhibit skeletal muscle degeneration, necrosis and interstitial fibrosis, as well as abnormal echocardiography results. By 15 weeks of age, the double homozygotes develop dilated cardiomyopathy. The double mutants exhibit kyphosis, compromised systolic and diastolic function, progressive fibrosis throughout the heart, cardiomyocyte membrane damage, ventricular cellular necrosis, and disorganized mitochondria and myofibrils in cardiomyocytes.

STATUS: requested; donating investigator able to send; line successfully re-derived at JAX December, 2012 and available for distribution May, 2012

4. JAX stock 013786 B6.129S1(Cg)-Lama2^{tm1Eeng}/J

Homozygous mice exhibit growth retardation and most die between 2-4 weeks of age. Laminin2, or Merosin, is expressed in striated muscle, peripheral and central nervous systems, thymus, thyroid, intestine, and testis and has been associated with merosin-deficient congenital muscular dystrophy (MCMD). Homozygous dy^w mice are passive, small, and emaciated, and demonstrate partial hindleg weakness and claspings. Their muscles contain necrotic fibers with

occasional areas of regeneration, and they exhibit pronounced fibrosis and increased serum creatine kinase (CK) activity. When heterozygous dy^W mice are bred with transgenic mice expressing the mouse muscle creatine kinase (Ckm or Mck) promoter driving the expression of human LAMA2 gene, the MCMD phenotype was rescued. These transgenic dy^W mice still become increasingly paralyzed with age and develop hindlimb contractures similar to nontransgenic dy^W mice likely due to failure of rescue in non-muscle tissues. These dy^W mutant mice are useful for studying merosin deficiency in MCMD and other muscular dystrophies.

STATUS: requested, donating investigator able to send; line successfully re-derived at JAX and expect complete backcross December, 2012. Available for distribution November, 2011.

Disseminate MD mouse and information resources to the scientific community through the MD Repository. JAX

- a) All mice that are available from the MD repository at JAX are readily accessed from our public website at JAX. Each strain has its own public datasheet with a description of the mouse, the development of the model, links to genotyping protocols, and animal husbandry information. For example <http://jaxmice.jax.org/strain/013786.html>
- b) We are working closely with a congenital muscle disease consortium that brings together families, advocates, scientists and clinicians working on congenital myopathy and congenital muscular dystrophy to outline key priorities to build infrastructure, support preclinical projects, improve clinical trial readiness and move towards clinical trials. Members include, Anne Rutkowski Cure CMD; Claudia Mitchell representing LGMD2I, The Joshua Frase Foundation (focus: myotubular and centronuclear myopathy) and A Foundation Building Strength (focus: nemaline myopathy), as well as researchers, Alan Beggs and Kevin Campbell. The consortium has been instrumental in advising us on models and raising awareness to the MD research community.
- c) Rare and Orphan Disease Center at JAX: Our website video features Brian Denger from Parent Project DMD. <http://www.jax.org/video/index.php?vid=rare-disease> This website also links to a An article in the JAX Search Magazine that features Dr. Greg Cox and his research on MD and Brian Denger's two children, Patrick and Mathew who both have DMD.
- d) Dr. Cathleen Lutz, Director of the Mouse Models Repository attended the 2011 Muscle Study Group meeting in Rochester New York and gave a 45 minute presentation of the MD mouse resources at JAX and our efforts to bank and standardize MD models for research and preclinical trials.

Aim 2. Engineer mice expressing in-frame deletions of the human dystrophin cDNA to model patients receiving successful exon-skipping therapies. JAX.

We have successfully generated the three human dystrophin cDNA clones containing in-frame mutations (deletion of exons 44-45, 49-51, 48-53) along with a full-length wildtype cDNA clone. The cDNAs have been fully sequence verified and plasmid vectors containing each clone are prepared. As shown in **Figure 1**, we are currently cloning the dystrophin cDNAs downstream of the mouse Titin

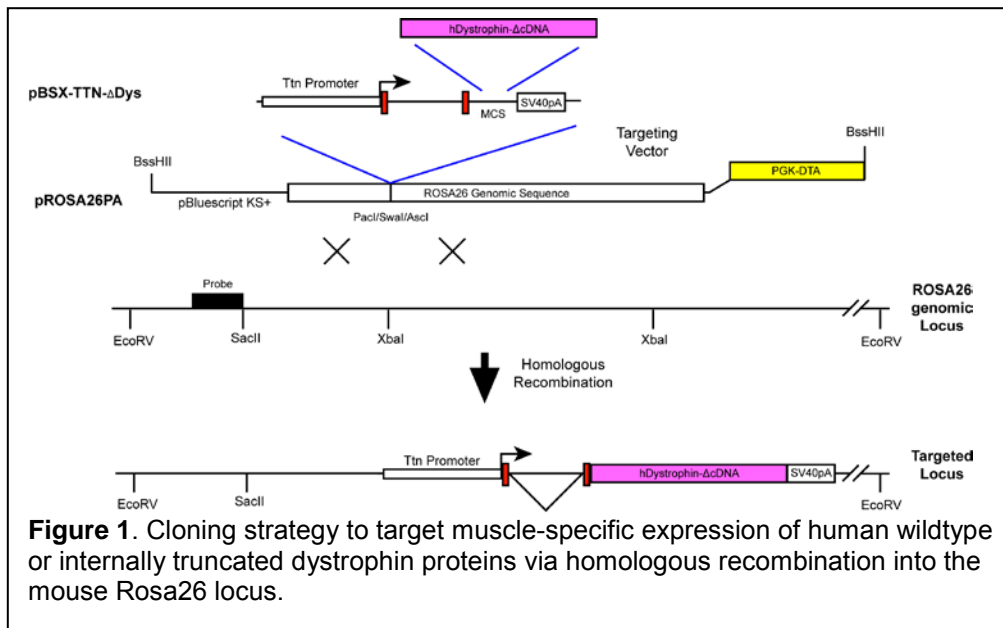


Figure 1. Cloning strategy to target muscle-specific expression of human wildtype or internally truncated dystrophin proteins via homologous recombination into the mouse Rosa26 locus.

(Ttn) promoter to drive high-level skeletal and cardiac muscle expression of the transgene. The Ttn promoter construct contains the first non-coding exon 1, the entire first intron and the 5' half of exon 2 truncated just upstream of the start codon. We have had great success using this promoter to drive skeletal and cardiac-specific expression of transgenes in the past and expect that a single-copy insertion into the Rosa26 locus should provide

uniform expression between each of the independent lines of mice generated by homologous recombination. Once the promoter/cDNA construct is completed in step 1, it will be inserted into a Rosa26 vector containing 5' and 3' homology arms for homologous recombination into ES cell lines.

Aim 3. Develop improved phenotypic *mdx* mouse models using genetic background variation in mice to map and identify genetic modifiers of disease severity. JAX.

Based on descriptions that the DMD^{mdx} mouse model had a more severe phenotype when crossed with a DBA mouse strain, we have completed the backcrossing of the *mdx* mutation onto the DBA/2J background using a speed congenic approach illustrated in **Figures 2 and 3**. The JAX DBA/2J substrain was chosen for this backcross as it was used by the Sanger Center for complete genomic sequencing to facilitate identification of genetic modifiers. The genetic quality control employed by the GRS at JAX during the marker-assisted backcross

ensures that the resulting mice contain >99.9% of the DBA/2J background across the genome.

Markers were chosen for genotyping out of 2,000+ markers in our set, spaced an average of approximately 1.5 Mb or 0.75cM apart and have been assayed on over 103 JAX® mouse strains,

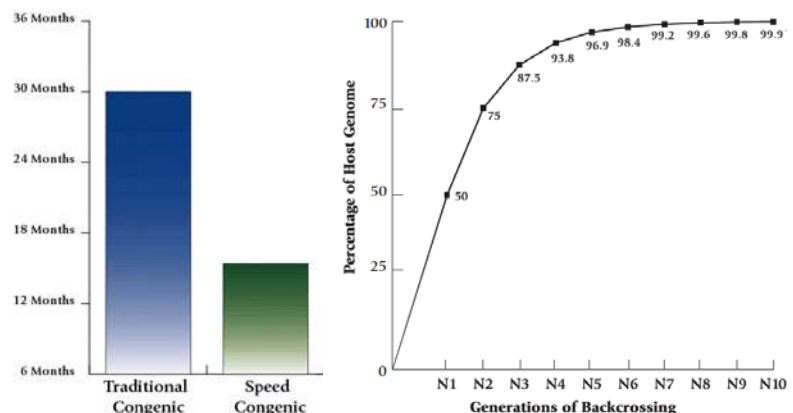


Figure 2. The time and number of traditional backcross generations needed to produce a fully congenic strain of mice (N10) with 99.9% of the host strain genome compared to a speed congenic approach.

including virtually all of the most commonly used JAX® inbred and wild-derived inbred strains. Intercrosses between D2.B10-+/mdx heterozygous N5 congenic mice are currently being used to generate homozygous mdx/mdx mice on the DBA/2J genetic background followed for phenotypic analysis. The backcrossing was slightly delayed due to small litter sizes of the DBA/2J background.

Aim 4. Test three promising therapeutic compounds (GW501516, AICAR and Dantrolene) in the preclinical DBA/2J-mdx congenic model. K. Nagaraju, CNMC.

In the present study, we have used agonists of PPAR δ (GW501516) and AMPK (AICAR) to activate beneficial endurance exercise-induced signaling pathways in *mdx* mice.

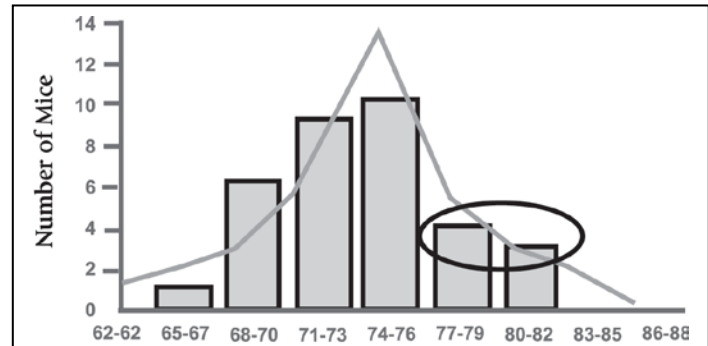


Figure 3. Percentage of genome expected to be DBA/2J-like after one backcross generation of B10-*mdx* to DBA/2J. The gray bell-shaped curve is the expected frequency and the bars represent the number of mice with each genotype. Mice with the highest percentage of D2 genome (circled) were selected for subsequent backcrossing to make N3 mice.

Effect of dystrophin deficiency on mitochondrial metabolism. Evaluation of mitochondrial mass (NAO staining) in EDL fibers of *mdx* mice and WT control mice (Fig. 1A) showed that dystrophin-deficient muscle fibers have decreased mitochondrial mass, indicating that these muscle have a lower capacity to use oxidative energy. Furthermore, assessment of LDH activity (Fig. 1B) demonstrated that the *mdx* mice had a greater capacity to produce lactate. These muscles also showed lower specific force than those of WT mice (Fig. 1C), and fatigue testing showed that dystrophin-deficient muscle was more fatigable than that of WT mice (Fig. 1D), suggesting that dystrophin deficiency leads to significant alterations in mitochondrial function and muscle metabolism.

Effect of GW and AICAR on muscle weight and behavioral activity measures. The average body mass of the treated mice was significantly higher than that of vehicle-treated mice (**Fig. 4A**).

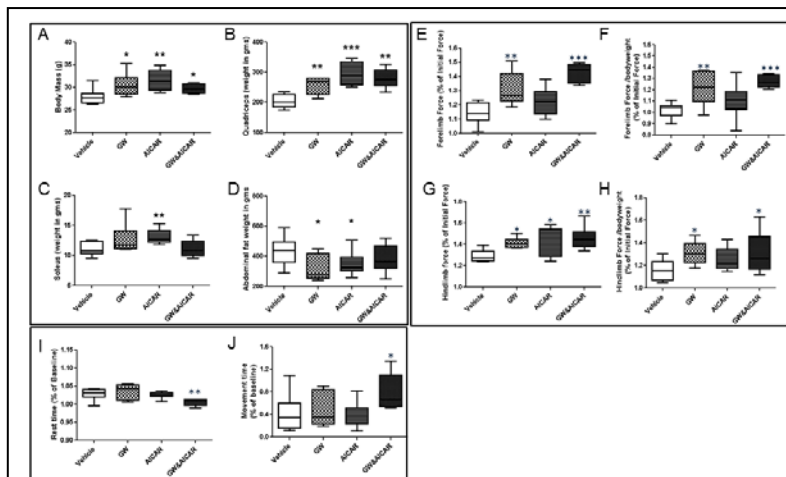


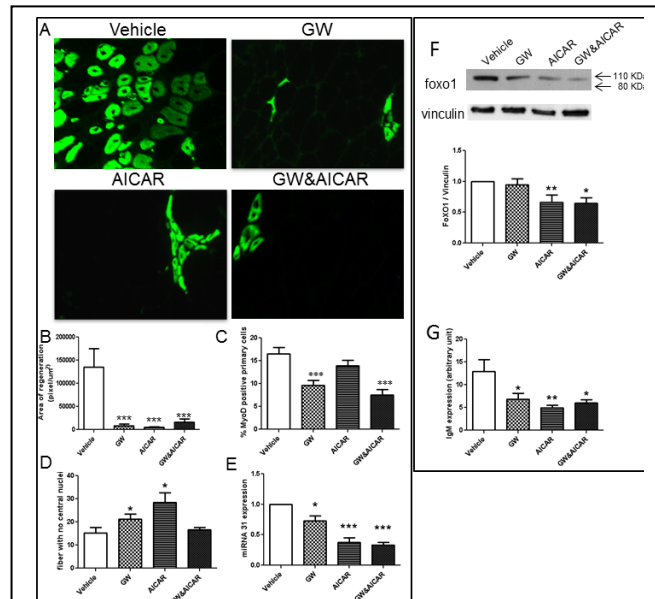
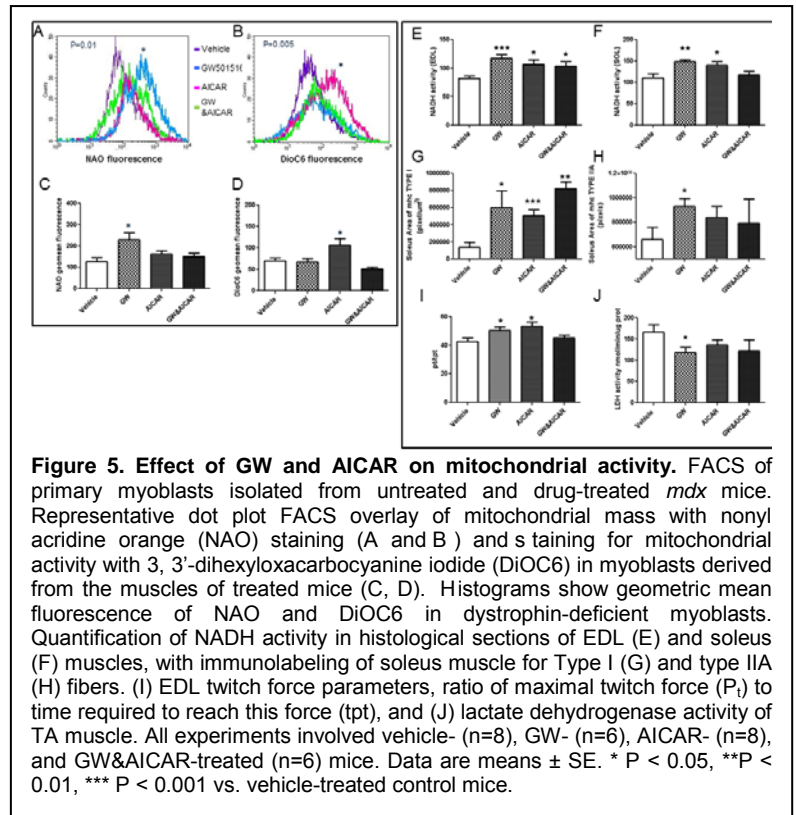
Figure 4. Effect of GW and AICAR on body mass, muscle mass, and behavioral activity. (A-D) Mass of vehicle control- (n=8), GW- (n=6), AICAR- (n=8), and GW&AICAR-treated (n=6) mice: mass of whole body (A), quadriceps (B), soleus (C), and abdominal fat (D) after euthanasia. Grip strength was measured using a grid at 12 & 16 weeks of age: (E) Maximal forelimb grip strength, (F) normalized maximal forelimb strength, (G) maximal hind-limb grip strength, (H) normalized maximal hind-limb strength of all groups. Overall activity of mice was measured using the open-field Digiscan apparatus at 12 & 16 weeks of age: (I) movement and (J) rest time activity. Behavioral activity is presented as % of the initial activity measured at 12 weeks of age before treatment. Data are means \pm SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. vehicle-treated control mice.

Treatments increased the body mass by ~10% (GW, 9.2%; AICAR, 11.3%; GW&AICAR, 10.63%). A general increase in the weight of the EDL, gastrocnemius, quadriceps, soleus, and TA muscles was found in the drug-treated groups (statistically significant for the quadriceps [GW, +12.7%; AICAR, +14.6%; GW&AICAR, 13.7%] (**Fig. 4B**) and soleus (GW, +14.3%; AICAR, +17.4%) (**Fig. 4C**). Interestingly, abdominal fat was decreased in response to all three treatments (**Fig. 4D**), and the decrease was statistically significant for both single-treatment groups.

Grip strength and open-field animal activity tests were performed before and after drug treatment. We found a significant increase in forelimb grip strength in the GW501516-treated and combination-treatment groups (**Fig. 4E,F**). The increase in hindlimb grip strength was significant for all three treatments (**Fig. 4G**). Since these drugs influenced body weight, we normalized data to body weight. Both forelimb and hindlimb grip strength increased significantly with GW501516 (+19%, +13%, respectively) and combination treatment (+25%, +13%, respectively) (**Fig. 4F**). Behavioral activity measures did not significantly change for the single treatments but the combination treatment group showed significantly increased movement time (89%) (**Fig. 4I**) and decreased rest time (**Fig. 4J**), suggesting an overall beneficial effect on these parameters.

Effect of GW and AICAR on

mitochondrial activity. We evaluated the impact of these drugs on mitochondrial activity in muscle cells isolated from hindlimb muscles of dystrophin-deficient *mdx* mice. A significant increase in



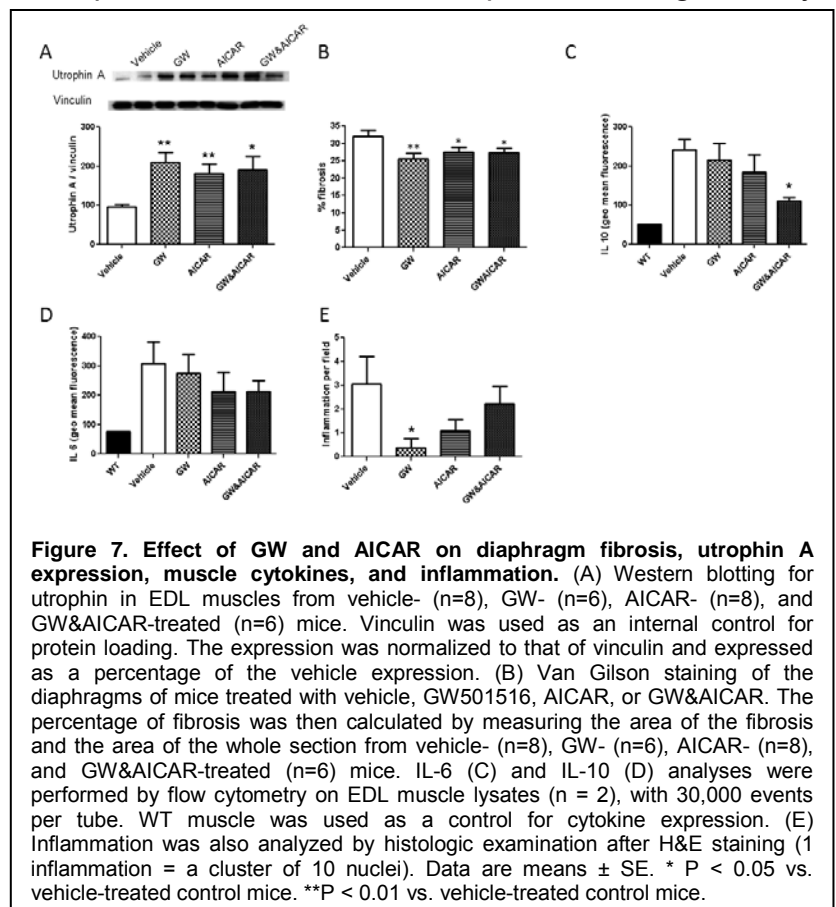
mitochondrial mass, as indicated by NAO staining, was found in the GW501516-treated cells (**Fig. 5A,B**). We saw no significant increase in either the AICAR- or combination-treated groups. Mitochondrial $\Delta\Psi$, as assessed by DiOC6 staining, was significantly increased in response to AICAR treatment (**Fig. 5C,D**) but not GW501516 or combination treatment. We also found that NADH activity was significantly increased in EDL (**Fig. 5E**) (GW, +43%; AICAR, +29%; GW&AICAR, 26%) and SOL (**Fig. 5E**) muscle (GW, +35%; AICAR, +26%; GW&AICAR, 13.7%) in response to drug treatment. Soleus muscles expressed more myosin heavy chain type I (**Fig. 5G**), whereas only GW showed a statistically significant increase in type IIA fibers (**Fig. 5H**). This increase in oxidative capacity was also observed in EDL muscles, in which the ratio of the height of the twitch force (P_t) to the time to reach this maximal force (tpt) was increased in single-treated mice (GW, +18%; AICAR, +24%) (**Fig. 5I**). Finally, LDH activity in the TA was decreased in all three treatment groups, but the decrease was only statistically significant for GW-treated mice (-30%) (**Fig. 5J**).

Effect of GW and AICAR on satellite cell activation and muscle regeneration and degeneration.

We found that the number of dMHC-positive fibers was significantly decreased in the drug-treated groups (**Fig. 6A&B**), and MyoD expression in isolated skeletal muscle cells was markedly decreased in the GW- and combination-treated groups (**Fig. 6C**). Furthermore, the number of EDL fibers without central nucleation increased in the single-treated groups (**Fig. 6D**). Importantly, miRNA31a expression, known to be associated with muscle regeneration/degeneration, was significantly down-regulated in diaphragms of treated mice (GW, -28%; AICAR, -63%; GW&AICAR, -67%) (**Fig. 6E**). miRNA133 was also increased in the treated groups, but the differences did not reach statistical significance (data not shown). Expression of FoXO1, which controls muscle wasting, was decreased in AICAR (-34%) and combination drug-treated mice (-36%) (**Fig. 6F**). Serum CK levels showed huge variations but no statistically significant changes (data not shown). Finally, IgM immunostaining was significantly decreased in gastrocnemius sections of treated muscle (GW, -48%; AICAR, -69%; GW&AICAR, -54%) (**Fig. 6G**). Overall, these data suggested a strong reduction in muscle degeneration.

Effect of GW and AICAR on diaphragm fibrosis, utrophin A expression, muscle cytokines, and inflammation.

Utrophin expression in skeletal muscle was studied because some of these improvements may have been due to utrophin expression¹⁷. The level of utrophin A was significantly increased in the treated groups over that in untreated mice (GW, +112.97%; AICAR, +84.97%; GW&AICAR, +94.19%) (**Fig. 7A**). We also measured fibrosis in the diaphragm which occurs early in the disease and serves as a useful marker for assessing disease progression and response therapy. We found that the red-positive area was significantly decreased in the treated groups (GW501516, -25.6%; AICAR, -27.5%; GW&AICAR, -27.2%) (**Fig. 7B**). Evaluation of cytokine expression in TA muscle lysates revealed that *mdx* mice had significantly increased IL-6 and IL-10 levels. Drug treatment did not significantly affect IL-6 expression (**Fig. 7C**), but IL-10 levels were significantly decreased in GW&AICAR-treated mice (-45%) (**Fig. 7D**), and not in individual drug-treated mice. EDL muscle demonstrated a statistically significant decrease in inflammatory infiltrates in the GW group but not the other two groups (**Fig. 7E**).



Summary: In summary, this study demonstrates that the use of endurance mimetics in *mdx* mice induces an improvement in the structural integrity and reduces the degeneration /regeneration of *mdx* mouse muscle, probably through an increase in oxidative metabolism in the fibers. Our study and other recent work underline the high potential of pharmacological activators of AMPK and PPAR δ as part of rational drug treatments for muscular dystrophies

C. KEY RESEARCH ACCOMPLISHMENTS

- We have established an MD Repository at The Jackson Laboratory that is actively soliciting critical models of human muscular dystrophy.
- We have imported and re-derived four lines of mice (Aim 1) into the MD Repository to add to our over 20 models of muscular dystrophy currently available.
- Genetic and phenotypic information regarding all new lines of mice have been posted to the JAX website and will soon be consolidated into a dedicated page for muscular dystrophy models.
- We have generated full-length and internally truncated human dystrophin cDNA clones for inclusion in targeted transgenic experiments that will test the potential efficacy of antisense oligonucleotide therapies to induce deletions of exons 44-45, 49-51, 48-53.
- We have created a D2.B10-mdx congenic strain of mice using a speed congenic protocol.
- We have shown that mitochondrial mass is significantly reduced in EDL fibers of *mdx* mice versus WT control mice, indicating that these muscles have a lower capacity to use oxidative energy.
- We have used agonists of PPAR δ (GW501516) and AMPK (AICAR) to activate beneficial endurance exercise-induced signaling pathways in *mdx* mice as a therapeutic strategy.
- We have shown that the use of endurance mimetics in *mdx* mice induce an improvement in the structural integrity and reduces the degeneration/regeneration of *mdx* mouse muscle, likely due to an increase in oxidative metabolism in the fibers.

D. REPORTABLE OUTCOMES

Abstracts and presentations

- | | |
|------|--|
| 2011 | Muscle Study Group meeting, Rochester, NY. C Lutz, JAX |
| 2011 | Parent Project Muscular Dystrophy, West Coast CONNECT meeting, San Diego, CA. K. Nagaraju, CNMC |
| 2011 | Myositis/Myopathy study group at the ACR annual meeting, Washington DC. K. Nagaraju, CNMC |
| 2011 | LGMD2A workshop sponsored by the Coalition to cure calpain3, Santa Monica, CA. K. Nagaraju, CNMC |
| 2011 | Fourth annual programs in Clinical and Translational Research: Muscular Dystrophy and Rehabilitation Medicine Programs in Clinical and Translational Research: Muscular Dystrophy and Rehabilitation Medicine, Washington DC. K. Nagaraju, CNMC |
| 2011 | Fifth annual dysferlin conference, Chicago, IL. K. Nagaraju, CNMC |
| 2011 | Oral presentation at the Jain foundation meeting. Chicago, IL. "Role of Toll-Like Receptors (TLRs) in the Pathogenesis of Dysferlin Deficiency." K. Nagaraju, CNMC |
| 2012 | 2012 international conference, organized by Duchenne parent project, Italy in Feb 17th-Feb19th Rome, Italy. K. Nagaraju, CNMC |

- 2012 32nd European Workshop for Rheumatology Research (EWRR) on February 23rd-25th, 2012, in Stockholm. **K. Nagaraju, CNMC**
- 2012 Myomatrix 2012 meeting at University of Nevada, Reno, April 22nd-24th 2012. **G Cox, JAX and K. Nagaraju, CNMC.**

E. CONCLUSION:

The development of an MD Repository will significantly increase the availability of high-demand strains of mice for research and will allow standardization of genetic background and genetic quality control to facilitate academic and pharmaceutical adoption of these strains for translational studies. We have begun outreach into the muscular dystrophy research and clinical community (congenital muscle disease consortium) to determine the models most anticipated for preclinical research and we are actively soliciting those models for inclusion in the repository. In Aim 2, our transgenic experiments to express in-frame deleted forms of dystrophin will model the best-case-scenario outcome for AO-mediated therapy in which one assumes that a particular compound is capable of 100% effective exon-skipping to restore the reading frame. By eliminating all of the caveats regarding the efficiency of delivery and pharmacodynamics of the particular therapeutic, we can provide a model that will allow functional analysis of the extent of phenotypic rescue for the three most common in-frame deletions for which clinical information from human Becker muscular dystrophy patients (deletion of exons 44-45, 49-51, 48-53) is lacking. In Aim 3, we have created a novel D2.B10-mdx congenic strain of mice that may serve as better preclinical model of disease should it show an increase severity of myopathic symptoms. These congenic mice will also provide the starting point for a genetic modifier screen to identify genes and pathways that affect disease severity. In addition, we have shown that endurance mimetics such as agonists of PPAR δ (GW501516) and AMPK (AICAR) are promising targets for treating Duchenne muscular dystrophy.